Differential effects of variations in human P450 oxidoreductase on the aromatase activity of CYP19A1 polymorphisms R264C and R264H

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Abstract: Aromatase converts androgens into estrogens and is required for female sexual development as well as growth and development in both sexes. Aromatase, coded by the CYP19A1 gene, is a member of cytochrome P450 family of heme-thiolate monoxygenases. Located in the endoplasmic reticulum, aromatase depends on reducing equivalents from reduced nicotinamide adenine dinucleotide phosphate through cytochrome P450 oxidoreductase coded by POR. Both the CYP19A1 and POR genes are highly polymorphic, and mutations in both these genes are linked to disorders of steroid biosynthesis. We have previously shown that R264C and R264 mutations in CYP19A1, as well as mutations in POR, result in a reduction of aromatase activity. The R264C is a common polymorphic variant of CYP19A1, with high frequency in Asian and African populations. Polymorphic alleles of POR are found in all population studied so far and therefore, may influence activities of CYP19A1 allelic variants. So far, effects of POR variations on activities of allelic variants of CYP19A1 or any other steroid metabolizing cytochrome P450 have not been studied. Here we are reporting the effects of several POR variants on the aromatase activities of CYP19A1 variants R264C and R264H. Using bacterially expressed and purified preparations of WT and variant forms of CYP19A1 and POR, we constructed liposomes with embedded CYP19A1 and POR proteins and assayed the activities using radiolabeled androstenedione as substrate. With the WT-POR as redox partner, the R264C-CYP19A1 showed only 15% of aromatase activity, but the R264H had 87% of aromatase activity compared to WT-CYP19A1. When the with P284L-POR as redox partner R264C-CYP19A1 lost all activity but retained 6.7% of activity when P284T-POR was used as redox partner. The R264H-CYP19A1 showed low activities with both the POR-284L as well as POR-284T. When POR-Y607C was used as redox partner, the R264C-CYP19A1 retained around 5% of aromatase activity. Remarkably, The R264H-CYP19A1 had more than three-fold higher activity compared to WT-CYP19A1 when POR-Y607C was used as the redox partner, pointing towards a beneficial effect. The slight increase in activity of R264C-CYP19A1 with P284T-POR and the three-fold increase in activity of R264H-CYP19A1 with Y607C-POR point towards a conformational effect and role of protein-protein interaction governed by R264C and R264H substitutions in CYP19A1 as well as P284L, P284T and Y607C variants of POR. These studies demonstrate that allelic variants of P450 when present with a variant form of POR may show different activities and combined effects of variations in both the P450 enzymes as well as in POR should be considered when genetic data are available. Recent trends in the whole exome and whole genome sequencing as diagnostic tools will permit combined evaluation of variations in multiple genes that are interdependent and may guide treatment options by adjusting therapeutic interventions based on laboratory analysis.

Keywords: Aromatase; POR, P450 oxidoreductase, CYP19A1, polymorphisms, cytochrome P450.

1 Introduction

Aromatase (CYP19A1) regulates estrogen biosynthesis [1] by converting androgens into estrogens (EC: 1.14.14.14) [2, 3]. Aromatase is a 503 amino acid protein (NP_000094) encoded by the CYP19A1 gene (GeneID:1588, NCBI: NM_000103), located on chromosome 15 (15q21.2, GRCh38 15:51208056-51338597). CYP19A1 is a membrane-bound protein located in the endoplasmic reticulum (ER) and is a member of the cytochrome P450 superfamily. Cytochrome P450 enzymes are involved in the biosynthesis of steroid hormones, and they also metabolize drugs and xenobiotics [4]. There are two different types of cytochrome P450 in humans. The type 1 P450 proteins are located in the mitochondrion and are primarily involved in the metabolism of steroid hormones and sterols [5, 6]. The type 2 P450 proteins are located in the endoplasmic reticulum and have a range of metabolic activities including drugs, xenobiotics as well as endogenous substrates, including steroid hormones like progesterone, dehydroepiandrosterone and testosterone [7-12]. Genetic defects in steroid metabolizing cytochromes P450 and their redox partners cause metabolic disorders with disordered steroidogenesis [13-19]. CYP19A1 is highly expressed in ovaries and plays a significant role in the regulation of the reproductive cycle in females [20]. In males, CYP19A1 is expressed in the gonads, and paracrine effects of aromatase products are essential for normal spermatogenesis [21-24]. Additionally, aromatase expression is also found in extra-gonadal tissues, including liver, muscle, placenta, bone, breast, adipose tissue, and brain [20, 25, 26]. Initial work on the characterization of aromatase was done on enzyme preparations purified from placenta by laboratories of Hall [27], Vickery [28] and Harada [29] and a placental preparation was also used for the determination of X-ray crystal structure of aromatase by Ghosh [30]. Aromatase is required for the synthesis of estrogens (C18 steroids) from androgen precursors (C19 steroids) (Figure 1) [1, 31]. Some of the critical reactions catalyzed by aromatase include the conversion of androstenedione into estrone (E1), testosterone to 17β-estradiol (E2) and 16-hydroxytestosterone to estriol (E3) [1]. The androstenedione and testosterone are well known and most common physiological steroid substrates for aromatase [1, 32, 33]. Recently 16β-OH-androstenedione was also reported to be a substrate for aromatase [34]. The catalytic process of aromatization of androgens is multifaceted, and it comprises of three steps involving the transfer of three pairs of electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and consumption of oxygen. Electron transfer from NADPH to aromatase is carried out by NADPH cytochrome P450 oxidoreductase (POR) [35, 36]. The first two steps of aromatase reaction are two sequential hydroxylations of the 19-methyl group of androgens whereas the last step causes the aromatization of the A-ring of substrate, which is specific for aromatase [37-39].

Multiple genetic defects are associated with decreased aromatase expression in both male and female reproduction (OMIM: 107910, 613546, 139300) [14, 40]. In females, it affects maternal virilization during gestation, abnormal external genitalia in the female fetus, normal ovarian development but increased polycystic ovary syndrome occurrence and virilization at puberty [25, 32]. The first case of placental aromatase deficiency was reported by Shozu *et al.* [2]. Later Morishima *et al.* found that aromatase deficiency manifests during fetal life in both sexes [41]. Impact of aromatase deficiency on growth, skeletal maturation, and bone homeostasis, [21, 42] as well as changes in insulin resistance and abnormal plasma lipid profile, have been described in earlier studies [14, 24, 25, 32, 43-46]. Estrogen excess has also been shown is multiple reports linked to increased aromatase expression and activity [47, 48]. Several natural compounds and including nicotine, vitamin E, and resveratrol may inhibit aromatase activity [49, 50]. Aromatase activity is also inhibited by bisphenol A and sildenafil [49]. Aromatase inhibitors like anastrozole are used in the treatment of breast cancer and to treat excess estrogen production in men [51].

POR deficiency (PORD, OMIM: 613537 and 201750) is a form of congenital adrenal hyperplasia, initially described in patients with altered steroidogenesis [19] followed by several reports with a broad spectrum of disorders [36, 52-56]. While many different cytochromes P450 have been tested with different POR variants, allelic variants of steroid metabolizing cytochromes P450 have not been studied for the potential changes in enzyme activities caused by variant forms of POR [36, 55, 57]. Subramanian et al had examined the combined effect of P450 and POR variations in a report describing activities of three common alleles of CYP2C9 assayed with WT and variant forms of POR

[58]. Since POR is obligate electron donor of all P450 proteins in the endoplasmic reticulum, variations in POR should also be taken into account when studying the effect of genetic polymorphisms in cytochromes P450. Disruption of CYP19A1 activity may have a severe impact on estrogen formation, and several variations of POR exist with significant population distribution, which can alter enzymatic activities of aromatase [59-63]. We, therefore, tested the impact of some population variants of POR on estrogen formation by the two variants of CYP19A1. R264C (rs700519) is a common polymorphic variant of CYP19A1(CYP19A1*4) while R264H (rs2304462) is a rare variant. We produced WT and mutant versions of human POR and human CYP19A1 as recombinant proteins and used liposome reconstitution to perform enzymatic assays and kinetic studies of CYP19A1-aromatase activity using androstenedione as a substrate. We also studied the effect of CYP19A1 variations on protein stability by limited proteolysis to understand a potential cause of change in enzyme activities by different variants. This study shows that variations in POR may have variable effects on activities of CYP19A1 variants, potentially leading to altered steroid metabolism in individuals with variant CYP19A1 alleles.

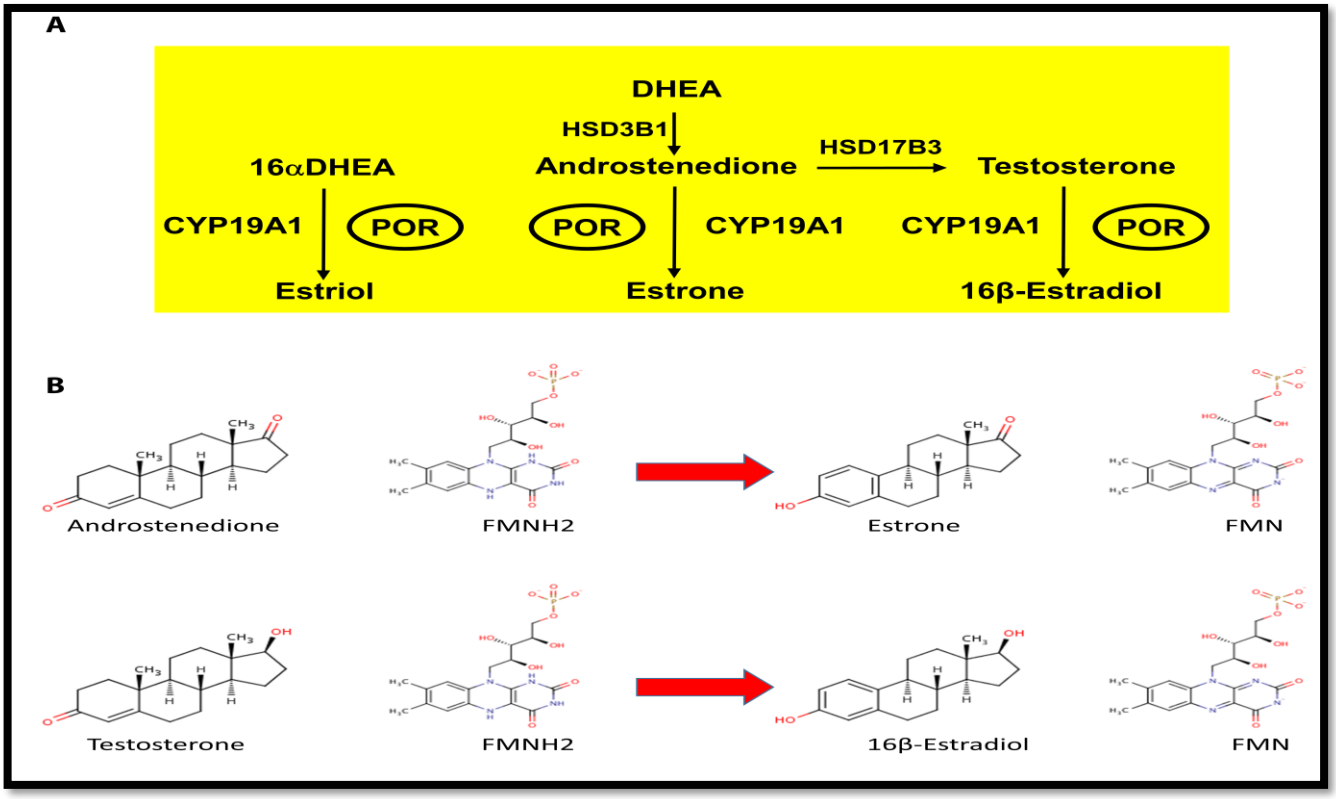

Figure 1: Steroid hormone biosynthesis and role of POR. Upper panel: In the placenta, DHEA (CHEBI:28689) is converted to androstenedione and then to testosterone (CHEBI:17347), which is further metabolized into estrogens. In fetal liver, inactive precursor DHEAS (CHEBI:16814) is converted to 16αDHEAS (CHEBI:87774), which gets converted to 16αDHEA in the placenta to produce estriol (CHEBI:27978". The estrogen biosynthesis process requires CYP19A1 activity that is dependent on POR. Lower panel: Electron transfer from NADPH to CYP19A1 is carried out via POR through reduced FAD and FMN. Chemical structures obtained from ChEBI (https://www.ebi.ac.uk/chebi/).

2 Materials and methods

2.1 Materials

Tris-base, NADPH, acetic acid, magnesium acetate, Sucrose, potassium phosphate EDTA, DTT, glycerol, PMSF, and Benzonase were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Carbenicillin, $FeCl_3$, $ZnCl_2$, $CoCl_2$, Na_2MoO_4, $CaCl_2$, $CuCl_2$, M H_3BO_3 were purchased from CarlRoth GmBH (Switzerland). Goat anti-rabbit antibodies labeled with infra-red dyes were from LI-COR Bioscience Inc. (NE, USA). The RC-DC protein assay dye reagent was from Bio-Rad, (Hercules, CA). The anti-POR antibody was from Genscript (NJ, USA).

2.2 Recombinant expression and purification of CYP19A1 and its polymorphic variants R264H and R264C

The experiments were carried out on a well-characterized recombinant soluble human CYP19A1, which has structure and activity comparable to full-length CYP19A1 [64]. Polymorphic variants R264H and R264C of CYP19A1 were generated as described previously by Baravalle *et al.* in 2017 [65]. CYP19A1 and the two polymorphic variants, R264H and R264C, were expressed and purified based previously described methods [64, 66-68]. Briefly, E.coli DH5α rubidium-competent cells were transformed with a pCWOri+ plasmid vector carrying the CYP19A1 gene and selected with 100 µg/mL ampicillin. CYP19A1 expression was induced by addition of 1 mM IPTG. Liquid cultures were grown in Terrific Broth medium in the presence of the heme precursor δ-aminolevulinic acid at 28°C for 48 hours. Cells were harvested and re-suspended in 100 mM potassium phosphate pH 7.4, 20 % glycerol, 1 % Tween-20 and 1 mM β-mercaptoethanol supplemented with 1 mg/mL lysozyme and protease inhibitor cocktail tablet (Roche, Switzerland) at 4°C. Cells were disrupted by sonication and ultra-centrifuged at 40,000 rpm for 20 minutes. The supernatant containing the soluble protein fraction was loaded onto a diethylaminoethyl ion-exchange column (DEAE-Sepharose Fast-Flow, GE Healthcare), followed by a Nickel-ion affinity column (Chelating-Sepharose Fast-Flow, GE Healthcare). The protein was eluted from the column with a gradient of 1 mM to 40 mM histidine, which was removed by ultrafiltration on Amicon Ultra 30,000 MWCO devices (Millipore).

2.3 POR expression and membrane purification

The POR WT and variants were expressed in bacteria using the expression constructs described previously [63, 69-71]. The protocol for the expression of POR variants and membrane as well as liposome preparation is reported in our earlier publications [59-61, 63, 69, 72]. WT or mutant POR constructs in pET22b were transformed into Escherichia coli BL21(DE3) and incubated at 37 °C overnight. Next day, single colonies were selected for growth on ampicillin, and grown in terrific broth media containing 40 mM $FeCl_3$, 4 mM $ZnCl_2$, 2 mM $CoCl_2$, 2 mM Na_2MoO_4, 2 mM $CaCl_2$, 2 mM $CuCl_2$, 2 mM H_3BO_3, 10% (v/v) potassium phosphate solution (0.17 M KH_2PO_4, 0.72 M K_2HPO_4 pH 7.4), 0.5 mg/ml of riboflavin and 100 µg/ml of carbenicillin at 37 °C to an optical density (OD) 600 nm of 0.6 and then the temperature was reduced to 25 °C and incubation was continued for 16 h. The bacteria were collected by centrifugation, washed with PBS and suspended in 100 mM Tris-acetate (pH 7.6), 0.5 M sucrose, and 1 mM EDTA and treated with lysozyme (0.5 mg/ml) and EDTA (0.1mM [pH 8.0]) at 4 ◦C for 1 h with occasional stirring to generate spheroplasts. The spheroplasts were collected in 50 ml tubes by centrifugation at 5000×g for 15 min; resuspended in a buffer preparation of 100 mM potassium phosphate (pH 7.6) containing 6 mM MgOAc, 0.1 mM DTT, 20% (v/v) glycerol, 0.2 mM PMSF, and 0.1 mM DNase I; and ruptured by an ultrasonic disruptor. A clear bacterial lysate devoid of cellular debris was obtained by high-speed centrifugation at 12,000×g for 10 min, and then the membranes were collected by ultracentrifugation at 100,000×g for 60 min at 4 ◦C. Membranes were suspended in 50mM Potassium phosphate buffer (pH 7.8) and 20% (v/v) glycerol and stored at −70 ºC. The concentration of protein preparations was measured by RC-DC protein assay (Protein Assay Dye Reagent, Bio-Rad, Hercules, CA) and the content of POR membranes was quantitated by western blot analysis.

2.4 Aromatase activity measurement in the reconstituted liposome system

Aromatase activity was measured by the tritiated water release assay based on an earlier method described by Lephart and Simpson [73] with modifications described previously [59, 63] using a reconstituted lipid-CYP19A1-POR system and androstenedione (androst-4-ene-3,17-dione) as the substrate. Bacterial membranes containing POR and purified CYP19A1 was reconstituted into DLPC-DLPG liposomes. Reaction mixture consisted of 50 pmol of CYP19A1, 200 pmol of POR and tritium labeled androstenedione ([1^{β}-^{3}H(N)]-andros-tene-3,17-dione; ~20,000 cpm) in 100 mM potassium phosphate buffer (pH 7.4) with 100 mM NaCl. Different concentrations (0.01–30 µM) of androstenedione were used for kinetic analysis. The aromatase reaction was started by addition of 1 mM NADPH and was incubated for 1 h under shaking

condition. The reaction was stopped adding chloroform. The water phase was mixed with 5% charcoal/0.5% dextran solution and centrifuged at 13000 rpm for 5 minutes. Finally, 0.5 ml supernatant was used for ^{3}H radioactivity counting. The specific activity of human aromatase was calculated as (pmol of product/minute of incubation/nmol of P450), where pmol of the product formations were calculated as:

$[cpm_{sample} - cpm_{blank}] \cdot$ pmol of cold androstenedione / Total cpm

Data were fitted based on Michaelis-Menten kinetics using GraphPad Prism (GraphPad Software, La Jolla, CA USA) to determine Vmax and Km. Values obtained from triplicate experiments are expressed as mean ± S.E.M. (standard error mean).

2.5 FastPP assay of CYP19A1 and its polymorphic variants R264H and R264C

The assay was performed as previously described [61, 74, 75]. Reactions mixtures contained 0.2 mg/mL of CYP19A1 WT or polymorphic variants R264H and R264C, 10 mM $CaCl_2$, 0.05 mg/mL thermolysis in 0.1 M KPi pH 7.4, 20% glycerol, 0.1 % Tween-20, 1 mM β-mercaptoethanol. The negative control was carried out without thermolysin. Digestion was performed for one minute in a TGradient Thermoblock (Biometra), generating a temperature gradient from 30 to 70°C, and stopped with 10 mM EDTA pH 8.0. Samples were analyzed by SDS-PAGE and quantified using Bio-Rad's Image Lab software.

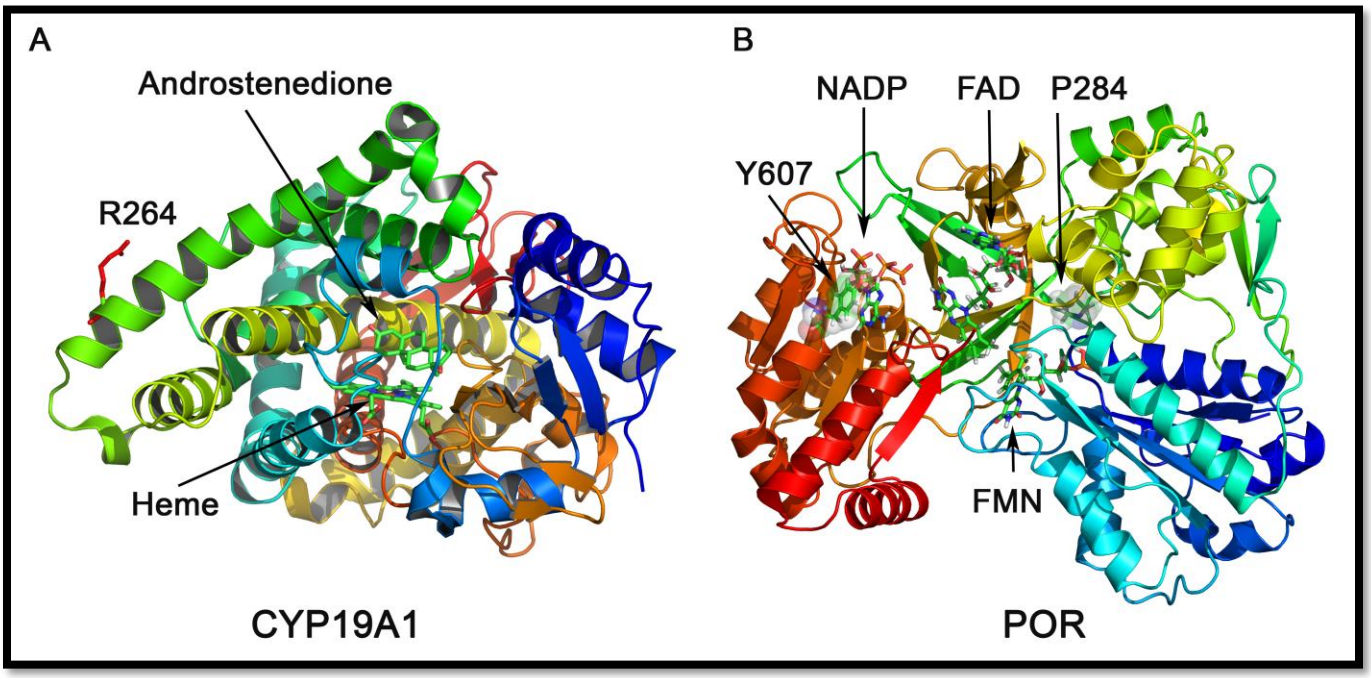

Figure 2: Location of CYP19A1 and POR variants: A. Location of CYP19A1 variants. The R264 residue on aromatase is surface exposed and located on the G helix of the CYP19A1 structure, which is part of the substrate access channel and it has been widely reported to be flexible in cytochromes P450. Here a ribbons model of CYP19A1 protein is shown and the bound substrate, androstenedione is depicted as sticks model. **B.** Location of POR variants. The model based on the x-ray crystal structure of the human POR (**PDB#3QE2**) showing the location of mutations studied in this report. The co-factors FMN, FAD, and NADP are shown as sticks. The mutation The P284 residue is located in the hinge region of the POR. The hinge region of provides conformational flexibility, and mutations in the hinge region would result in conformational changes that would alter the interaction of POR with its redox partners.

3 Results

3.1 Genetic sequence analysis of CYP19A1 variants R264C and R264H.

The R264C variant of CYP19A1 is present with a lower frequency in the European population with a MAF of 0.02 in 1000 Genomes database (Table 1). Among the American population, the MAF of R264C was 0.04. In the East Asian (MAF 0.17), South East Asian (MAF 0.24) and African (MAF 0.18) populations the R264C variant was present at much higher frequency. Looking individually, the Italian population from Tuscany had lowest occurrence of R264C MAF of 0.006 in HapMap database (Table 1). In the HapMap database the of R264C was found in Mexican population with a MAF of 0.01, while Japanese population showed the highest presence of R264C with a MAF 0f 0.27.

HapMap data showed similar MAF among subpopulations populations as reported in 1000 Genome database, the only exception being Maasai population from Kenya which had R264C present at MAF of 0.04, which is much lower than the distribution in other African populations.

Table 1: Genetic data for the CYP19A1 variations. The R264C (DBSNP: rs700519) is a common polymorphism in the CYP19A1 gene. Population codes referred in the table are for **1000 Genome**: **AMR** American; **AFR** African; **EAS** East Asian; **SAS** South East Asian; **EUR** European. For the **HapMap** project: **ASW**, African ancestry in Southwest USA; **CEU**, Utah residents with Northern and Western European ancestry; **CHB**, Han Chinese in Beijing, China; **CHD**, Chinese in Metropolitan Denver, Colorado; **GIH**, Gujarati Indians in Houston, Texas; **JPT**, Japanese in Tokyo, Japan; **LWK**, Luhya in Webuye, Kenya; **MEX**, Mexican ancestry in Los Angeles, California, USA; **MKK**, Maasai in Kinyawa, Kenya; **TSI**, Toscans in Italy; **YRI**, Yoruba in Ibadan, Nigeria. The R264H (rs2304462) is a rare variant and has been seen with lower frequencies across populations. Data for population genetic distribution of variants were obtained from the NCBI genome database (http://www.ncbi.nlm.nih.gov). Populations with minor allele frequency significantly higher than average are shown in bold.

Population ID-Class	Number of alleles	Major Allele Freq.	Minor Allele Freq.
R264C (rs700519)			
1000 Genome	5008	G=0.860	A=0.140
1000 Genome- AMR	694	G=0.96109998	A=0.03890000
1000 Genome-AFR	1322	G=0.81770003	A=0.18230000
1000 Genome-EAS	1008	G=0.82940000	A=0.17060000
1000 Genome-SAS	978	G=0.76279998	**A=0.23720001**
1000 Genome-EUR	1006	G=0.97119999	A=0.02880000
ESP-cohort North America	4542	G=0.91391456	A=0.08608542
HapMap			
HapMap-CEU	224	G=0.96875000	A=0.03125000
HapMap -TSI	174	G=0.99425286	A=0.00574713
HapMap -MEX	98	G=0.98979592	A=0.01020408
HapMap -CHD	170	G=0.88235295	A=0.11764706
HapMap -JPT	172	G=0.72674417	**A=0.27325583**
HapMap -ASW	98	G=0.83673471	A=0.16326530
HapMap -GIH	174	G=0.83908045	A=0.16091955
HapMap -CHB	82	G=0.85365856	A=0.14634146
HapMap -YRI	224	G=0.78571427	**A=0.21428572**
HapMap -LWK	180	G=0.88888890	A=0.11111111
HapMap -MKK	286	G=0.95804197	A=0.04195804
R264H (rs2304462)			
ExAc Aggregated Populations	121366	C=0.99996704	T=0.00003296
gnomAD-Exomes	246062	C=0.99998	T=0.00002

3.2 Genetic distribution of POR variants.

The POR variants studied in this report have been identified by mining the genome databases and performing structural stability and sequence conservation analysis to find out which of the variants may be potentially disease-causing [54]. Human POR is a 680 amino acid protein which has evolved from ferredoxin and ferredoxin reductase-like domains to form a single redox protein in eukaryotes. All variations in POR studied here are highly conserved across species and are found as rare variants in NCBI Exome database (Table 2). The P284L is a rare variant of predominantly European origin and the P284T had been only observed in African population. The Y607C is rare variant seen in European and Asian populations (Table 2).

POR	DBSNP id	Global	European	Asian	American	African
P284L	rs72557938	T=0.00005	T=0.00004	T=0.0001	T=0.0000	T=0.0000
P284T	rs72557937	A=0.00004	A=0.00000	A=0.0000	A=0.0000	A=0.0005
Y607C	rs72557954	G=0.00011	G=0.00008	G=0.0003	G=0.0000	G=0.0000

Table 2: Minor allele Frequencies of POR variants obtained from the NCBI Exome database.

3.3 Structural analysis of CYP19A1 and POR variants

The R264 residue on aromatase is surface exposed and located on the G helix of the CYP19A1 structure (Figure 2A), which is part of the substrate access channel and it has been widely reported to be flexible in cytochromes P450. R264 is the central residue of a 3 Arg-containing cluster that is part of a consensus sequence for different kinases. Thus, it can play a key role in both the regulation of substrate access and aromatase activity by post-translational modifications (51).

The P284 residue is located in the hinge region of the POR (Figure 2B). The hinge region in POR provides conformational flexibility, and mutations in the hinge region would result in conformational changes that may alter the interaction of POR with its redox partners [36, 59]. The Y607 residue is located close to NADPH binding site (Figure 2B) and The Y607C mutation in POR has been shown to affect the aromatase activity [60].

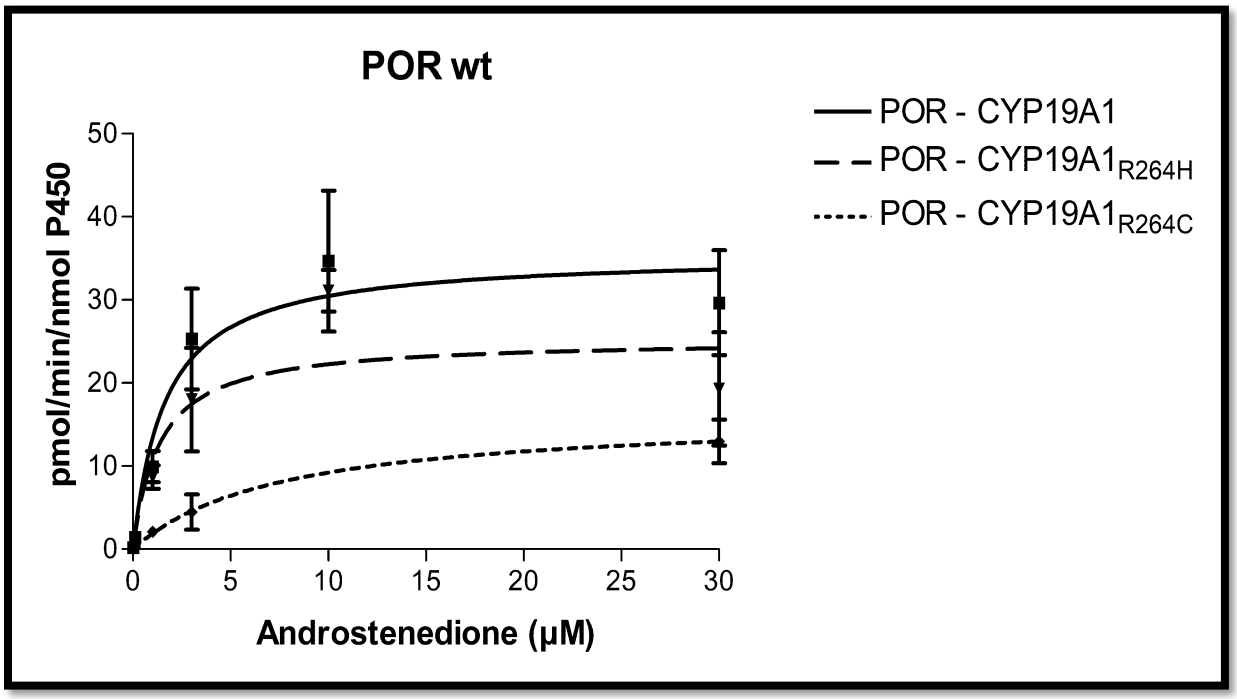

Figure 3: Aromatase activity of CYP19A1 variants with WT-POR. CYP19A1 wild type (solid line) and mutants $CYP19A1_{R264H}$ (dashed line) and $CYP19A1_{R264C}$ (dashed-dotted line) activities supported by POR-WT were tested to compare the activities of three different types of CYP19A1 variants. Recombinant preparations of purified human CYP19A1 (WT and variants) expressed in bacteria and recombinant human POR were incorporated into liposomes. Aromatase activity to convert tritium labeled androstenedione to estrone was measured by the tritiated water release assay. Data were fitted with Michaelis-Menten kinetics model using GraphPad Prism. The calculated Km and Vmax values are summarized in Table 3.

	Km, Androstenedione (µM)	Vmax pmol/min/nmol	Vmax/Km	% WT
CYP19A1; aromatase (androstenedione to estrone)				
CYP19A1-WT	1.6 ± 0.9	35.5 ± 4.7	21.6	100.0
CYP19A1-R264H	1.3 ± 0.9	25.2 ± 4.1	18.8	87.0
CYP19A1-R264C	5.1 ± 2.6	16.9 ± 2.8	3.3	15.3

Table 3. Kinetic parameters for activities of CYP19A1 and its polymorphic variants supported by WT POR. For the conversion of androstenedione to estrone, variable concentrations (0.3–30 µM) of androstenedione were used for kinetic analysis in the presence of 1 mM NADPH.

3.4 Aromatase activity of CYP19A1 and its polymorphic variants R264H and R264C supported by POR-WT

We performed *in-vitro* aromatase activity assay with wild type CYP19A1 and its polymorphic variants (R264H and R264C) to study the effect of two mutations on enzyme activity. To this aim, reconstituted liposomes were prepared by mixing purified recombinant CYP19A1 and purified membranes containing WT-POR as described in material and methods. Then we accessed the enzyme activity by standard tritiated water release assay. For the R264H variant of CYP19A1, the apparent Km for androstenedione was comparable to that of WT whereas the apparent Vmax was reduced by ~30% (Figure 3, Table 3). Therefore, the activity of CYP19A1-R264H variant was reduced by ~15% compared to WT CYP19A1. However, for R264C variant of CYP19A1, the apparent Km for androstenedione increased by 3.2 fold as compared to WT CYP19A1. The apparent Vmax was reduced by ~50%. Therefore, CYP19A1-R264C variant showed ~7 fold reduction in catalytic efficiency (Vmax/km) with only 15% residual aromatase activity as compared to WT CYP19A1 (Figure 3, Table 3). This loss of activity indicates that R264C mutation of CYP19A1 affects either the interaction of the substrate with CYP19A1 or the CYP19A1-POR interaction, which may influence electron transfer from POR to CYP19A1 for metabolic reactions.

	Km, Androstenedione (µM)	Vmax pmol/min/nmol	Vmax/Km	% WT
POR-P284L				
CYP19A1-WT	99.7 ± 312.0	37.4 ± 92.7	0.4	1.7
CYP19A1-R264H	55.2 ± 49.7	38.6 ± 24.0	0.7	3.2
CYP19A1-R264C	n.d.	n.d.	n.d.	n.d.
POR-P284T				
CYP19A1-WT	22.5 ± 36.2	15.8 ± 13.2	0.7	3.3
CYP19A1-R264H	51.0 ± 56.5	24.5 ± 18.7	0.5	2.2
CYP19A1-R264C	19.9 ± 184.2	28.7 ± 109.9	1.4	6.7
POR-Y607C				
CYP19A1-WT	4.7 ± 2.5	18.3 ± 3.0	3.9	18.2
CYP19A1-R264H	1.3 ± 0.6	17.7 ± 1.7	13.6	63.1
CYP19A1-R264C	77.5 ± 133.6	81.1 ± 105.0	1.0	4.8

Table 4. Kinetic parameters for activities of CYP19A1 and its polymorphic variants supported by variant forms of POR. For the conversion of androstenedione to estrone, variable concentrations (0.3–30 µM) of androstenedione were used for kinetic analysis in the presence of 1 mM NADPH.

3.5 Stability analysis of CYP19A1 and its polymorphic variants R264H and R264C by Fast Proteolysis Assay (FASTpp)

To compare the biophysical protein stability CYP19A1 and its polymorphic variants R264H and R264C, we applied a FASTpp to recombinantly expressed and purified proteins. CYP19A1-WT and variants were exposed in parallel to a range of different temperature with thermostable protease Thermolysin. Thermolysin readily cleaves the unfolded protein parts near the hydrophobic residues Phe, Leu, Ile and Val (42,43) but does not cleave the folded parts of the protein. Increasing the temperature induces thermal unfolding of proteins and the temperature-dependent changes in the degradation pattern represent the readout for the stability of the proteins. The SDS-PAGE analysis of the parallel FASTpp of CYP19A1-WT and variants (Figure 4A and B) showed that the R264H and R264C mutation does not affect protein stability. Therefore, it seems likely that conformational changes induced by R264C and R264H mutations does not significantly alter the structural stability of CYP19A1. However, the changes caused by these mutations may alter the delicate balance of protein-protein interactions that are needed for transfer of electrons from POR to CYP19A1.

3.6 Variable effects on Aromatase activity of CYP19A1 and its polymorphic variants R264H and R264C are shown by POR variants

To study how different variants of CYP19A1 interact with variants of POR; we tested 3 polymorphic variants of POR (P284L, P284T, and Y607C) in supporting aromatase activity. We tested the *in-vitro* estrogen synthase activity of aromatase by tritiated water release assay with reconstituted liposomes using androstenedione as a substrate. The POR variants P284L and P284T showed severe loss of aromatase activity with all CYP19A1 variants tested (Figure 5A-B, Table 4). For POR-P284L variant, the apparent Km for androstenedione was increased ~60 fold and ~35 fold as compared to WT POR with CYP19A1 and CYP19A1-R264H. The apparent Vmax of POR-P284L was comparable to that of POR-WT in supporting CYP19A1 and CYP19A1-R264H activity. However, the reaction with POR-P284L and CYP19A1-R264C was undetectable, and Km and Vmax could not be determined. For POR-P284T variant, the apparent Km for androstenedione was increased ~14, ~32 and ~12 fold with CYP19A1, CYP19A1-R264H, and CYP19A1-R264C respectively. The apparent Vmax was reduced by ~55%, ~30%, and ~20% with CYP19A1, CYP19A1-R264H and CYP19A1-R264C, respectively.

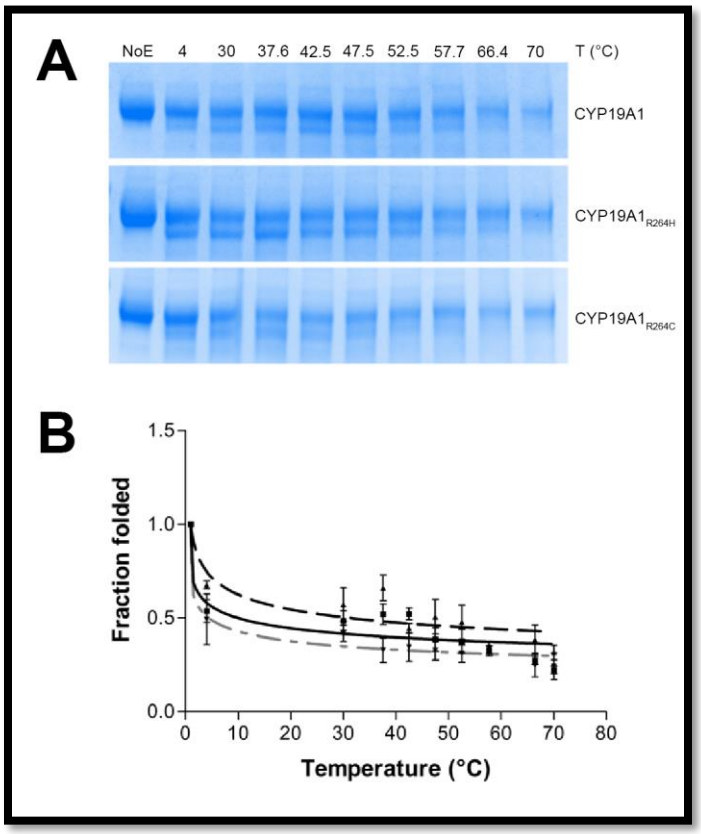

Figure 4: FastPP assay for the stability of CYP19A1 variants. Protein stability analysis of aromatase mutants $CYP19A1_{R264H}$ (dashed line) and $CYP19A1_{R264C}$ (dashed-dotted line) compared to CYP19A1-WT (solid line) by FASTpp assay. Thermolysin (0.05) mg/mL) was used to digest 0.20 mg/mL of CYP19A1-WT or mutants at increasing temperatures ranging from 4°C to 70°C. NoE represents the negative control carried out without thermolysin. The experiment was repeated at least three times, and a representative SDS-PAGE is shown.

3.7 POR-Y607C variant supports CYP19A1-R264H better than WT CYP19A1.

For POR-Y607C variant, the apparent Km for androstenedione was increased ~3 and ~48 fold with CYP19A1 and CYP19A1-R264C respectively (Figure 5C, Table 4). The apparent Vmax was reduced by ~50%, with both CYP19A1 and CYP19A1-R264H. Therefore, the residual activity of CYP19A1, CYP19A1-R264H, and CYP19A1-R264C with POR-Y607C was 18.2%, 63.1 and 4.8% respectively as compared to POR-WT: CYP19A1 activity. Interestingly, POR-Y607C variant supports CYP19A1-R264H better than WT CYP19A1.

Discussion

POR is obligate electron donor for all P450 enzymes in the endoplasmic reticulum and supplies electrons for the catalytic activities of aromatase. Aromatase deficiency is linked to genetic disorders of estrogen biosynthesis in women and developmental disorders in men. Previously we have studied CYP19A1 variants (R264C and R26H) and POR genetic polymorphisms in studies that focused on individual genes [54, 59, 65]. Cytochromes P450 and P450 reductase have a large number of genetic variations, and therefore, many individuals may harbor variations in P450 reductase as well as P450 genes that may alter the enzymatic activities of cytochromes P450 [76]. So far combined effects of variations in P450 reductase and steroidogenic P450 have not been tested, despite many reports on effects of genetic variations that were tested separately, using WT P450 proteins with variant forms of POR; or P450 variants tested with WT-POR. There is only one report in literature describing the effects of genetic variations in a P450 protein (CYP2C9) with some POR variations [58].

In this report, we have tested the combinations of genetic variants in CYP19A1, responsible for estrogen production, with variant forms of POR. The R264C is a common polymorphism in CYP19A1 with a higher frequency in Asian and African populations compared to American, Mexican, or European populations. The only exception was found in the Maasai population from Kinyawa in Kenya, where the R264C variant of CYP19A1 was present at a frequency seem among European or American populations (Table 1). The prevalence of R264C variant of CYP19A1 is high in Japanese, Chines, and South East Asian population. Among the POR variant studied, The Y607C has also been observed in the South East Asian population [54]. In the aromatase activity assay using bacterially expressed recombinant forms of POR and CYP19A1 proteins, we studied the combined effects of POR and CYP19A1 variant in different permutations and combinations of two proteins. First, we tested the variant forms of CYP19A1 with WT POR and found that the R264C variant lost around 85% of aromatase activity, while the activity of R264H variant was decreased by 14% when compared to WT-CYP19A1. These data are in line with the previous report where the two polymorphic variants showed a decrease in the catalytic efficiency compared to the WT protein [51]. However, in this work the amount of activity loss was different and this can be explained with a different experimental set up, due to the incorporation of the enzymes in liposomes, the use of higher amounts of POR (4:1 *versus* 1:1 ratio) and the different aromatase activity assay.

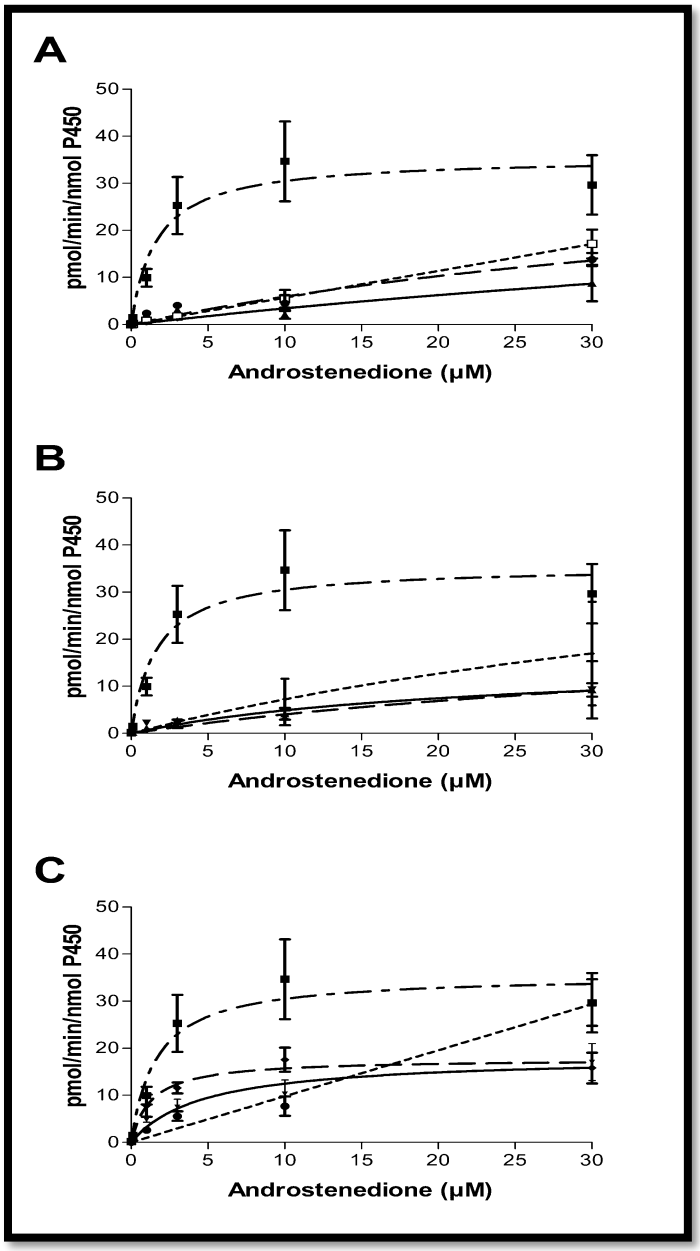

Figure 5: Aromatase activity. WT-CYP19A1(solid line) and mutants R264H -CYP19A1 (dashed line) and R264C -CYP19A1 (dotted line) activities supported by A: POR-P284L, B: POR-P284T, C: POR-Y607C. Aromatase activity supported by the wild type POR is shown in all three graphs as a reference (dash-dotted line). Human CYP19A1 (WT and variants R264C/R264H) and human POR (WT and variants P284L/P284T/Y607C) were embedded into liposomes, and aromatization activity to convert for the conversion of androstenedione to estrone was monitored by the tritiated water release assay. Data were fitted with Michaelis-Menten kinetics model using GraphPad Prism. The calculated Km and Vmax values are summarized in Table 3 and Table 4.

POR is a membrane associated protein that binds NADPH, causing a conformation change that brings NAHPH and FAD close together to transfer electrons. Afterwards, further conformation changes cause a closing of the POR structure and brings FAD and FMN closer for electron transfer from FAD to FMN. The FMN binding domain of POR interacts with P450s and other partner proteins through charge pair interactions involving arginine and lysine residues on P450s and aspartate and glutamate residues on POR [77-80]. Considering the importance of hinge region of POR in protein-protein interactions we investigated the effects of mutations in this region of POR for enzymatic activities of aromatase variants. A main reason behind such sensitivity of CYP19A1 towards changes in POR may be due to its requirement of six pairs of electrons to complete the conversion of androgens (androstenedione, testosterone) to estrogens (estrone, estradiol). By this logic it is possible that changes in conformation of POR, that affect interactions with CYP19A1, could in turn affect aromatase activities. The P284L (rs 72557938) and P284T (rs72557937) mutations are located in the hinge region of POR, that is important for domain movements and efficient interactions between FMN and FAD binding regions and for the transfer of electrons from FAD to FMN (Figure 2B). In the sequencing study by Miller laboratory the P284L mutation was present at an allele frequency of 0.003 in Chinese American population [71] and in the currently available ExAc dataset containing 121000 samples it is present at an allele frequency of 0.00004132. A 54% loss of 17-hydroxylase activity was reported by Huang et al but the 17,20 lyase activity of CYP17A1 was 82% of the wild type POR [71]. Recently we have shown that the P284L variant of POR showed reduced activities with several drug metabolizing cytochromes P450 [76]. There was 55% loss of activity in CYP2C19 and CYP3A5 assays while 78% loss of activity was observed in CYP2C9 assays with the P284L variant of POR compared to the WT protein [76]. No loss of flavin content was observed in P284L variant of POR [59] which indicates altered inter as well as intra molecular protein-protein interactions may be responsible for the loss of activities of CYP19A1. Since the hinge region of POR is important for the domain motions to bring the FMN closer to the FAD for the transfer of electrons, a change in this region may affect the efficiency of electron transport process in POR, and therefore, affect the activities of partner proteins [81]. It is possible that some redox partners of POR may experience greater impact on their catalytic activities while others may retain close to wild type activities depending on their mode of interaction with POR [82].

As seen with P284L, P284T and Y607C mutations, there may be variations of POR in apparently normal populations which result in disease causing effects. Since POR variations has now been linked to metabolism of several drugs and steroids, it may be prudent to include sequencing of POR gene while looking for genetic causes of altered drug or steroid metabolism [54, 55, 57, 59, 70]. A detailed examination of polymorphic variants of POR shall be conducted to check the potentially damaging effect of any variants.

Acknowledgments: This work was supported by grants from the Swiss National Science Foundation (31003A-135926), the Novartis Foundation for Medical-Biological Research (18A053) and Burgergemiende Bern to AVP and by CRT Foundation (project "Exposome" RF = 2016.2780) to GDN.

References

[1] E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, M.M. Hinshelwood, S. Graham-Lorence, B. Amarneh, Y. Ito, C.R. Fisher, M.D. Michael, et al., Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, Endocr Rev 15(3) (1994) 342-355.

[2] M. Shozu, K. Akasofu, T. Harada, Y. Kubota, A new cause of female pseudohermaphroditism: placental aromatase deficiency, J Clin Endocrinol Metab 72(3) (1991) 560-566.

[3] N. Harada, H. Ogawa, M. Shozu, K. Yamada, K. Suhara, E. Nishida, Y. Takagi, Biochemical and molecular genetic analyses on placental aromatase (P-450AROM) deficiency, J Biol Chem 267(7) (1992) 4781-4785.

[4] U.M. Zanger, M. Schwab, Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation, Pharmacol Ther 138(1) (2013) 103-141.

[5] A. Zalewski, N.S. Ma, B. Legeza, N. Renthal, C.E. Flück, A.V. Pandey, Vitamin D-Dependent Rickets Type 1 Caused by Mutations in CYP27B1 Affecting Protein Interactions With Adrenodoxin, J Clin Endocrinol Metab 101(9) (2016) 3409-3418.

[6] T. Omura, Mitochondrial P450s, Chem Biol Interact 163(1-2) (2006) 86-93.

[7] T. Omura, Contribution of cytochrome P450 to the diversification of eukaryotic organisms, Biotechnol Appl Biochem 60(1) (2013) 4-8.

[8] B.C. Chung, K.J. Matteson, W.L. Miller, Structure of a bovine gene for P-450c21 (steroid 21-hydroxylase) defines a novel cytochrome P-450 gene family, Proc Natl Acad Sci U S A 83(12) (1986) 4243-4247.
[9] B.C. Chung, K.J. Matteson, R. Voutilainen, T.K. Mohandas, W.L. Miller, Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta, Proc Natl Acad Sci U S A 83(23) (1986) 8962-8966.
[10] K.J. Matteson, B.C. Chung, M.S. Urdea, W.L. Miller, Study of cholesterol side-chain cleavage (20,22 desmolase) deficiency causing congenital lipoid adrenal hyperplasia using bovine-sequence P450scc oligodeoxyribonucleotide probes, Endocrinology 118(4) (1986) 1296-1305.
[11] B.C. Chung, J. Picado-Leonard, M. Haniu, M. Bienkowski, P.F. Hall, J.E. Shively, W.L. Miller, Cytochrome P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues, Proc Natl Acad Sci U S A 84(2) (1987) 407-411.
[12] Y. Morel, J. Picado-Leonard, D.A. Wu, C.Y. Chang, T.K. Mohandas, B.C. Chung, W.L. Miller, Assignment of the functional gene for human adrenodoxin to chromosome 11q13----qter and of adrenodoxin pseudogenes to chromosome 20cen----q13.1, Am J Hum Genet 43(1) (1988) 52-59.
[13] M. Fernandez-Cancio, N. Camats, C.E. Fluck, A. Zalewski, B. Dick, B.M. Frey, R. Monne, N. Toran, L. Audi, A.V. Pandey, Mechanism of the Dual Activities of Human CYP17A1 and Binding to Anti-Prostate Cancer Drug Abiraterone Revealed by a Novel V366M Mutation Causing 17,20 Lyase Deficiency, Pharmaceuticals 11(2) (2018).
[14] C.M. Pepe, N.I. Saraco, M.S. Baquedano, G. Guercio, E. Vaiani, R. Marino, A.V. Pandey, C.E. Fluck, M.A. Rivarola, A. Belgorosky, The cytochrome P450 aromatase lacking exon 5 is associated with a phenotype of nonclassic aromatase deficiency and is also present in normal human steroidogenic tissues, Clin Endocrinol (Oxf) 67(5) (2007) 698-705.
[15] M. Janner, A.V. Pandey, P.E. Mullis, C.E. Flück, Clinical and biochemical description of a novel CYP21A2 gene mutation 962_963insA using a new 3D model for the P450c21 protein, Eur J Endocrinol 155(1) (2006) 143-151.
[16] W.L. Miller, N. Huang, C.E. Flück, A.V. Pandey, P450 oxidoreductase deficiency, Lancet 364(9446) (2004) 1663.
[17] W.L. Miller, B.C. Chung, The First Defect in Electron Transfer to Mitochondrial P450 Enzymes, Endocrinology 157(3) (2016) 1003-1006.
[18] W.L. Miller, Minireview: regulation of steroidogenesis by electron transfer, Endocrinology 146(6) (2005) 2544-2550.
[19] C.E. Flück, T. Tajima, A.V. Pandey, W. Arlt, K. Okuhara, C.F. Verge, E.W. Jabs, B.B. Mendonca, K. Fujieda, W.L. Miller, Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome, Nat Genet 36(3) (2004) 228-230.
[20] S.E. Bulun, E.R. Simpson, Regulation of aromatase expression in human tissues, Breast cancer research and treatment 30(1) (1994) 19-29.
[21] J.P. Bilezikian, A. Morishima, J. Bell, M.M. Grumbach, Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency, N Engl J Med 339(9) (1998) 599-603.
[22] V. Rochira, A. Balestrieri, M. Faustini-Fustini, S. Borgato, P. Beck-Peccoz, C. Carani, Pituitary function in a man with congenital aromatase deficiency: effect of different doses of transdermal E2 on basal and stimulated pituitary hormones, J Clin Endocrinol Metab 87 (2002) 2857-2862.
[23] L. Lin, O. Ercan, J. Raza, C.P. Burren, S.M. Creighton, R.J. Auchus, M.T. Dattani, J.C. Achermann, Variable phenotypes associated with aromatase (CYP19) insufficiency in humans, J Clin Endocrinol Metab 92(3) (2007) 982-990.
[24] A. Belgorosky, G. Guercio, C. Pepe, N. Saraco, M.A. Rivarola, Genetic and clinical spectrum of aromatase deficiency in infancy, childhood and adolescence, Horm Res 72(6) (2009) 321-330.
[25] F.A. Conte, M.M. Grumbach, Y. Ito, C.R. Fisher, E.R. Simpson, A syndrome of female pseudohermaphrodism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450arom), J Clin Endocrinol Metab 78(6) (1994) 1287-1292.
[26] E.R. Simpson, Aromatase: biologic relevance of tissue-specific expression, Semin Reprod Med 22(1) (2004) 11-23.
[27] S. Nakajin, M. Shinoda, P.F. Hall, Purification to homogeneity of aromatase from human placenta, Biochem. Biophys. Res. Commun. 134 (1986) 704-710.
[28] J.T. Kellis, L.E. Vickery, Purification and characterization of human placental aromatase cytochrome P-450, J. Biol. Chem. 262 (1987) 4413-4420.
[29] N. Harada, Novel properties of human placental aromatase as cytochrome P-450: Purification and characterization of a unique form of aromatase., J Biochem (Tokyo) 103(1) (1988) 106-133.
[30] D. Ghosh, J. Griswold, M. Erman, W. Pangborn, Structural basis for androgen specificity and oestrogen synthesis in human aromatase, Nature 457(7226) (2009) 219-223.
[31] M.M. Grumbach, R.J. Auchus, Estrogen: consequences and implications of human mutations in synthesis and action, J Clin Endocrinol Metab 84(12) (1999) 4677-4694.
[32] C. Stocco, Tissue physiology and pathology of aromatase, Steroids 77(1-2) (2012) 27-35.
[33] G. Di Nardo, G. Gilardi, Human aromatase: perspectives in biochemistry and biotechnology, Biotechnol Appl Biochem 60(1) (2013) 92-101.
[34] J. Neunzig, M. Milhim, L. Schiffer, Y. Khatri, J. Zapp, A. Sanchez-Guijo, M.F. Hartmann, S.A. Wudy, R. Bernhardt, The steroid metabolite 16(beta)-OH-androstenedione generated by CYP21A2 serves as a substrate for CYP19A1, J Steroid Biochem Mol Biol 167 (2017) 182-191.
[35] M.B. Murataliev, R. Feyereisen, A. Walker, Electron transfer by diflavin reductases, Bba-Proteins Proteom 1698(1) (2004) 1-26.
[36] A.V. Pandey, C.E. Flück, NADPH P450 oxidoreductase: structure, function, and pathology of diseases, Pharmacol Ther 138(2) (2013) 229-254.
[37] P.J. Mak, A. Luthra, S.G. Sligar, J.R. Kincaid, Resonance Raman spectroscopy of the oxygenated intermediates of human CYP19A1 implicates a compound i intermediate in the final lyase step, J Am Chem Soc 136(13) (2014) 4825-4828.
[38] Y. Khatri, A. Luthra, R. Duggal, S.G. Sligar, Kinetic solvent isotope effect in steady-state turnover by CYP19A1

suggests involvement of Compound 1 for both hydroxylation and aromatization steps, FEBS Lett 588(17) (2014) 3117-3122.
[39] M. Akhtar, J.N. Wright, Acyl-Carbon Bond Cleaving Cytochrome P450 Enzymes: CYP17A1, CYP19A1 and CYP51A1, Adv Exp Med Biol 851 (2015) 107-130.
[40] N. Bouchoucha, D. Samara-Boustani, A.V. Pandey, H. Bony-Trifunovic, G. Hofer, Y. Aigrain, M. Polak, C.E. Flück, Characterization of a novel CYP19A1 (aromatase) R192H mutation causing virilization of a 46,XX newborn, undervirilization of the 46,XY brother, but no virilization of the mother during pregnancies, Mol Cell Endocrinol 390(1-2) (2014) 8-17.
[41] A. Morishima, M.M. Grumbach, E.R. Simpson, C. Fisher, K. Qin, Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens, J Clin Endocrinol Metab 80(12) (1995) 3689-3698.
[42] I. Czajka-Oraniec, E.R. Simpson, Aromatase research and its clinical significance, Endokrynol Pol 61(1) (2010) 126-134.
[43] P.E. Mullis, N. Yoshimura, B. Kuhlmann, K. Lippuner, P. Jaeger, H. Harada, Aromatase deficiency in a female who is compound heterozygote for two new point mutations in the P450arom gene: impact of estrogens on hypergonadotropic hypogonadism, multicystic ovaries, and bone densitometry in childhood, J Clin Endocrinol Metab 82(6) (1997) 1739-1745.
[44] M. Ludwig, A. Beck, L. Wickert, U. Bolkenius, B. Tittel, K. Hinkel, F. Bidlingmaier, Female pseudohermaphroditism associated with a novel homozygous G-to-A (V370-to-M) substitution in the P-450 aromatase gene, J Pediatr Endocrinol Metab 11(5) (1998) 657-664.
[45] J. Deladoey, C. Fluck, M. Bex, N. Yoshimura, N. Harada, P.E. Mullis, Aromatase deficiency caused by a novel P450arom gene mutation: impact of absent estrogen production on serum gonadotropin concentration in a boy, J Clin Endocrinol Metab 84(11) (1999) 4050-4054.
[46] L. Maffei, Y. Murata, V. Rochira, G. Tubert, C. Aranda, M. Vazquez, C.D. Clyne, S. Davis, E.R. Simpson, C. Carani, Dysmetabolic syndrome in a man with a novel mutation of the aromatase gene: effects of testosterone, alendronate, and estradiol treatment, J Clin Endocrinol Metab 89(1) (2004) 61-70.
[47] M. Shozu, S. Sebastian, K. Takayama, W.T. Hsu, R.A. Schultz, K. Neely, M. Bryant, S.E. Bulun, Estrogen excess associated with novel gain-of-function mutations affecting the aromatase gene, N Engl J Med 348(19) (2003) 1855-1865.
[48] C.A. Stratakis, A. Vottero, A. Brodie, L.S. Kirschner, D. DeAtkine, Q. Lu, W. Yue, C.S. Mitsiades, A.W. Flor, G.P. Chrousos, The aromatase excess syndrome is associated with feminization of both sexes and autosomal dominant transmission of aberrant P450 aromatase gene transcription, J Clin Endocrinol Metab 83(4) (1998) 1348-1357.
[49] R. Baravalle, F. Valetti, G. Catucci, G. Gambarotta, M. Chiesa, S. Maurelli, E. Giamello, I. Barone, S. Catalano, S. Ando, G. Di Nardo, G. Gilardi, Effect of sildenafil on human aromatase activity: From in vitro structural analysis to catalysis and inhibition in cells, J Steroid Biochem Mol Biol 165(Pt B) (2017) 438-447.
[50] R. Baravalle, A. Ciaramella, F. Baj, G. Di Nardo, G. Gilardi, Identification of endocrine disrupting chemicals acting on human aromatase, Biochimica et biophysica acta. Proteins and proteomics 1866(1) (2018) 88-96.
[51] G. Di Nardo, G. Cimicata, R. Baravalle, V. Dell'Angelo, A. Ciaramella, G. Catucci, P. Ugliengo, G. Gilardi, Working at the membrane interface: Ligand-induced changes in dynamic conformation and oligomeric structure in human aromatase, Biotechnol Appl Biochem 65(1) (2018) 46-53.
[52] M. Adachi, K. Tachibana, Y. Asakura, T. Yamamoto, K. Hanaki, A. Oka, Compound heterozygous mutations of cytochrome P450 oxidoreductase gene (POR) in two patients with Antley-Bixler syndrome, Am J Med Genet A 128A(4) (2004) 333-339.
[53] W. Arlt, E.A. Walker, N. Draper, H.E. Ivison, J.P. Ride, F. Hammer, S.M. Chalder, M. Borucka-Mankiewicz, B.P. Hauffa, E.M. Malunowicz, P.M. Stewart, C.H. Shackleton, Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study, Lancet 363(9427) (2004) 2128-2135.
[54] F.Z. Burkhard, S. Parween, S.S. Udhane, C.E. Flück, A.V. Pandey, P450 Oxidoreductase deficiency: Analysis of mutations and polymorphisms, J Steroid Biochem Mol Biol 165(Pt A) (2017) 38-50.
[55] A.V. Pandey, P. Sproll, Pharmacogenomics of human P450 oxidoreductase, Front Pharmacol 5 (2014) 103.
[56] C.E. Flück, A.V. Pandey, Steroidogenesis of the testis -- new genes and pathways, Ann Endocrinol (Paris) 75(2) (2014) 40-47.
[57] C.E. Flück, A.V. Pandey, Human P450 Oxidoreductase Deficiency, in: I. Huhtaniemi, L. Martini (Eds.), Encyclopedia of Endocrine Diseases (Second Edition), Academic Press, Oxford, 2019. pp. 431-443.
[58] M. Subramanian, V. Agrawal, D. Sandee, H.K. Tam, W.L. Miller, T.S. Tracy, Effect of P450 oxidoreductase variants on the metabolism of model substrates mediated by CYP2C9.1, CYP2C9.2, and CYP2C9.3, Pharmacogenet Genomics 22(8) (2012) 590-597.
[59] S.S. Udhane, S. Parween, N. Kagawa, A.V. Pandey, Altered CYP19A1 and CYP3A4 Activities Due to Mutations A115V, T142A, Q153R and P284L in the Human P450 Oxidoreductase, Frontiers in Pharmacology 8 (2017).
[60] C.E. Flück, A.V. Pandey, Impact on CYP19A1 activity by mutations in NADPH cytochrome P450 oxidoreductase, J Steroid Biochem Mol Biol 165(Pt A) (2017) 64-70.
[61] S. Parween, F. Roucher-Boulez, C.E. Flück, A. Lienhardt-Roussie, D. Mallet, Y. Morel, A.V. Pandey, P450 Oxidoreductase Deficiency: Loss of Activity Caused by Protein Instability From a Novel L374H Mutation, J Clin Endocrinol Metab 101(12) (2016) 4789-4798.
[62] C.E. Flück, D. Mallet, G. Hofer, D. Samara-Boustani, J. Leger, M. Polak, Y. Morel, A.V. Pandey, Deletion of P399_E401 in NADPH cytochrome P450 oxidoreductase results in partial mixed oxidase deficiency, Biochem Biophys Res Commun 412(4) (2011) 572-577.
[63] A.V. Pandey, P. Kempna, G. Hofer, P.E. Mullis, C.E. Flück, Modulation of human CYP19A1 activity by mutant NADPH P450 oxidoreductase, Mol Endocrinol 21(10) (2007) 2579-2595.
[64] J. Lo, G. Di Nardo, J. Griswold, C. Egbuta, W. Jiang, G. Gilardi, D. Ghosh, Structural basis for the functional roles of

critical residues in human cytochrome p450 aromatase, Biochemistry 52(34) (2013) 5821-5829.
[65] R. Baravalle, G. Di Nardo, A. Bandino, I. Barone, S. Catalano, S. Ando, G. Gilardi, Impact of R264C and R264H polymorphisms in human aromatase function, J Steroid Biochem Mol Biol 167 (2017) 23-32.
[66] N. Kagawa, Efficient expression of human aromatase (CYP19) in E. coli, Methods Mol Biol 705 (2011) 109-122.
[67] N. Kagawa, H. Hori, M.R. Waterman, S. Yoshioka, Characterization of stable human aromatase expressed in E. coli, Steroids 69(4) (2004) 235-243.
[68] N. Kagawa, Q. Cao, K. Kusano, Expression of human aromatase (CYP19) in Escherichia coli by N-terminal replacement and induction of cold stress response, Steroids 68(2) (2003) 205-209.
[69] N. Huang, A.V. Pandey, V. Agrawal, W. Reardon, P.D. Lapunzina, D. Mowat, E.W. Jabs, G. Van Vliet, J. Sack, C.E. Flück, W.L. Miller, Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis, Am J Hum Genet 76(5) (2005) 729-749.
[70] C. Nicolo, C.E. Flück, P.E. Mullis, A.V. Pandey, Restoration of mutant cytochrome P450 reductase activity by external flavin, Mol Cell Endocrinol 321(2) (2010) 245-252.
[71] N. Huang, V. Agrawal, K.M. Giacomini, W.L. Miller, Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations, Proc Natl Acad Sci U S A 105(5) (2008) 1733-1738.
[72] A.V. Pandey, C.E. Flück, P.E. Mullis, Altered heme catabolism by heme oxygenase-1 caused by mutations in human NADPH cytochrome P450 reductase, Biochem Biophys Res Commun 400(3) (2010) 374-378.
[73] E.D. Lephart, E.R. Simpson, Assay of aromatase activity, Methods Enzymol 206 (1991) 477-483.
[74] M.C. Miletta, A. Eble, M. Janner, S. Parween, A.V. Pandey, C.E. Fluck, P.E. Mullis, IGHD II: A Novel GH-1 Gene Mutation (GH-L76P) Severely Affects GH Folding, Stability, and Secretion, J Clin Endocrinol Metab 100(12) (2015) E1575-1583.
[75] D.P. Minde, M.M. Maurice, S.G. Rudiger, Determining biophysical protein stability in lysates by a fast proteolysis assay, FASTpp, PLoS One 7(10) (2012) e46147.
[76] M.N.R. Velazquez, S. Parween, S.S. Udhane, A.V. Pandey, Variability in human drug metabolizing cytochrome P450 CYP2C9, CYP2C19 and CYP3A5 activities caused by genetic variations in cytochrome P450 oxidoreductase, Biochem Biophys Res Commun 519 (2019) 133-138.
[77] M. Sugishima, H. Sato, K. Wada, K. Yamamoto, Crystal structure of a NADPH-cytochrome P450 oxidoreductase (CYPOR) and heme oxygenase 1 fusion protein implies a conformational change in CYPOR upon NADPH/NADP+ binding, FEBS Letters 593(8) (2019) 868-875.
[78] R. Bernhardt, K. Pommerening, K. Ruckpaul, Modification of Carboxyl Groups on Nadph-Cytochrome-P-450 Reductase Involved in Binding of Cytochromes-C and P-450 Lm2, Biochem Int 14(5) (1987) 823-832.
[79] R. Bernhardt, R. Kraft, K. Ruckpaul, Molecular Mechanism of Cytochrome-P-450 Reductase Interaction, Cytochrome P-450 : Biochemistry and Biophysics / (1989) 320-323.
[80] R. Bernhardt, R. Kraft, A. Otto, K. Ruckpaul, Electrostatic Interactions between Cytochrome-P-450 Lm2 and Nadph-Cytochrome-P-450 Reductase, Biomed Biochim Acta 47(7) (1988) 581-592.
[81] K. Bavishi, D.R. Li, S. Eiersholt, E.N. Hooley, T.C. Petersen, B.L. Moller, N.S. Hatzakis, T. Laursen, Direct observation of multiple conformational states in Cytochrome P450 oxidoreductase and their modulation by membrane environment and ionic strength, Scientific Reports 8 (2018).
[82] J.P. Connick, J.R. Reed, W.L. Backes, Characterization of Interactions Among CYP1A2, CYP2B4, and NADPH-cytochrome P450 Reductase: Identification of Specific Protein Complexes, Drug Metabolism and Disposition 46(3) (2018) 197-203.